SUPPLEMENTAL DATA

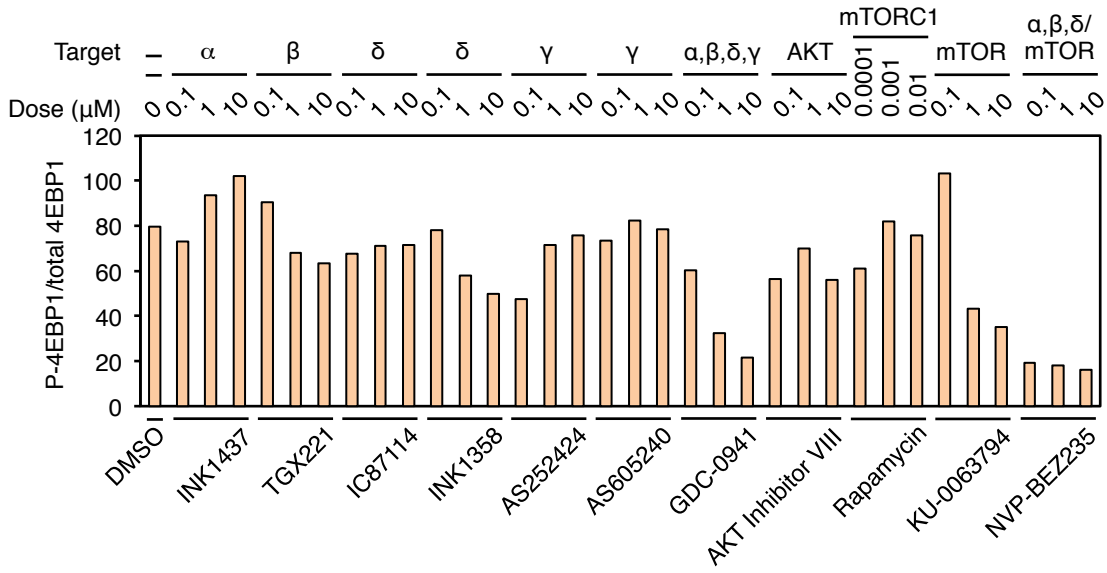
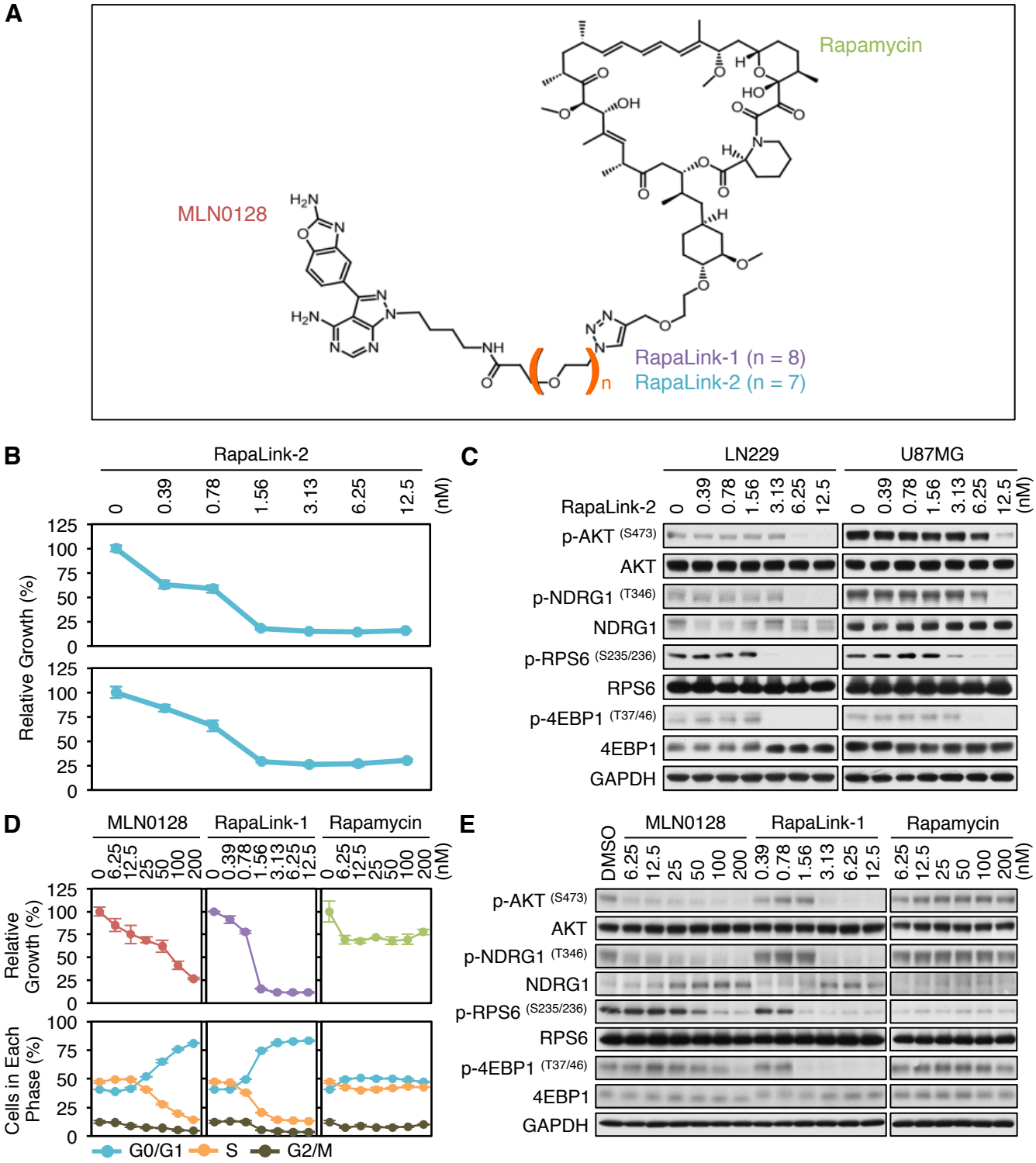
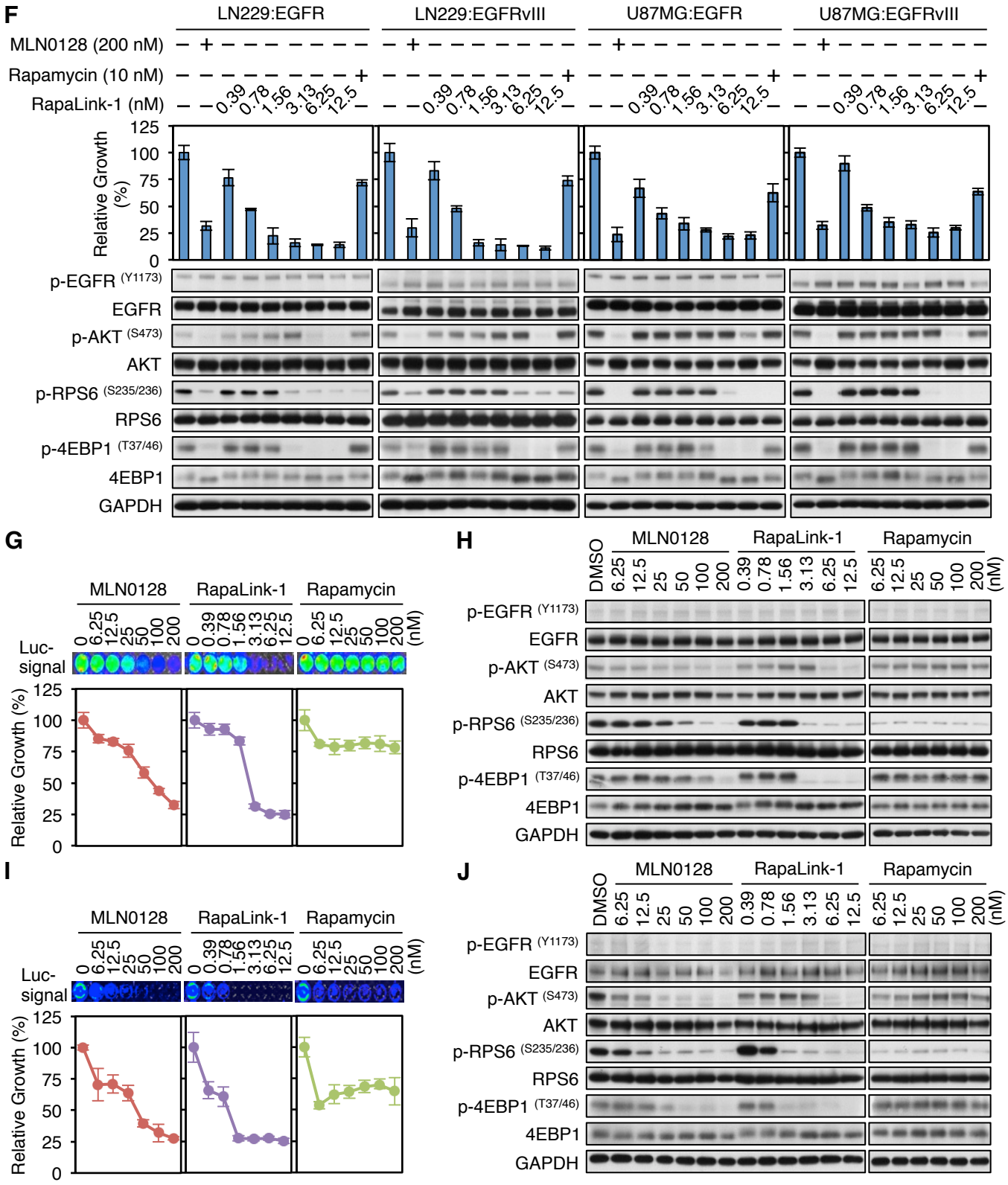


Figure S1 (related to Figure 1). Abundance of p-4EBP1$^{T37/46}$ and total 4EBP1 following treatment of LN229 cells with inhibitors.

The band intensity of p-4EBP1$^{T37/46}$ and total 4EBP1 from Figure 1D was quantified by densitometry using Silver Fast Scanner and ImageJ software. The ratios of phosphorylation to total protein are presented.


A
Rapamycin
MLN0128
RapaLink-1 (n = 8)
RapaLink-2 (n = 7)
B
RapaLink-2
0
0.39
0.78
1.56
3.13
6.25
12.5
(nM)
Relative Growth (%)
C
LN229
U87MG
RapaLink-2
p-AKT (S473)
AKT
p-NDRG1 (T346)
NDRG1
p-RPS6 (S235/236)
RPS6
p-4EBP1 (T37/46)
4EBP1
GAPDH
D
MLN0128
RapaLink-1
Rapamycin
Relative Growth (%)
Cells in Each Phase (%)
G0/G1
S
G2/M
E
DMSO
MLN0128
RapaLink-1
Rapamycin
p-AKT (S473)
AKT
p-NDRG1 (T346)
NDRG1
p-RPS6 (S235/236)
RPS6
p-4EBP1 (T37/46)
4EBP1
GAPDH


F
LN229:EGFR
LN229:EGFRvIII
U87MG:EGFR
U87MG:EGFRvIII
MLN0128 (200 nM)
Rapamycin (10 nM)
RapaLink-1 (nM)
0.39
0.78
1.56
3.13
6.25
12.5
Relative Growth (%)
p-EGFR (Y1173)
EGFR
p-AKT (S473)
AKT
p-RPS6 (S235/236)
RPS6
p-4EBP1 (T37/46)
4EBP1
GAPDH
G
MLN0128
RapaLink-1
Rapamycin
Luc-signal
H
DMSO
I
J

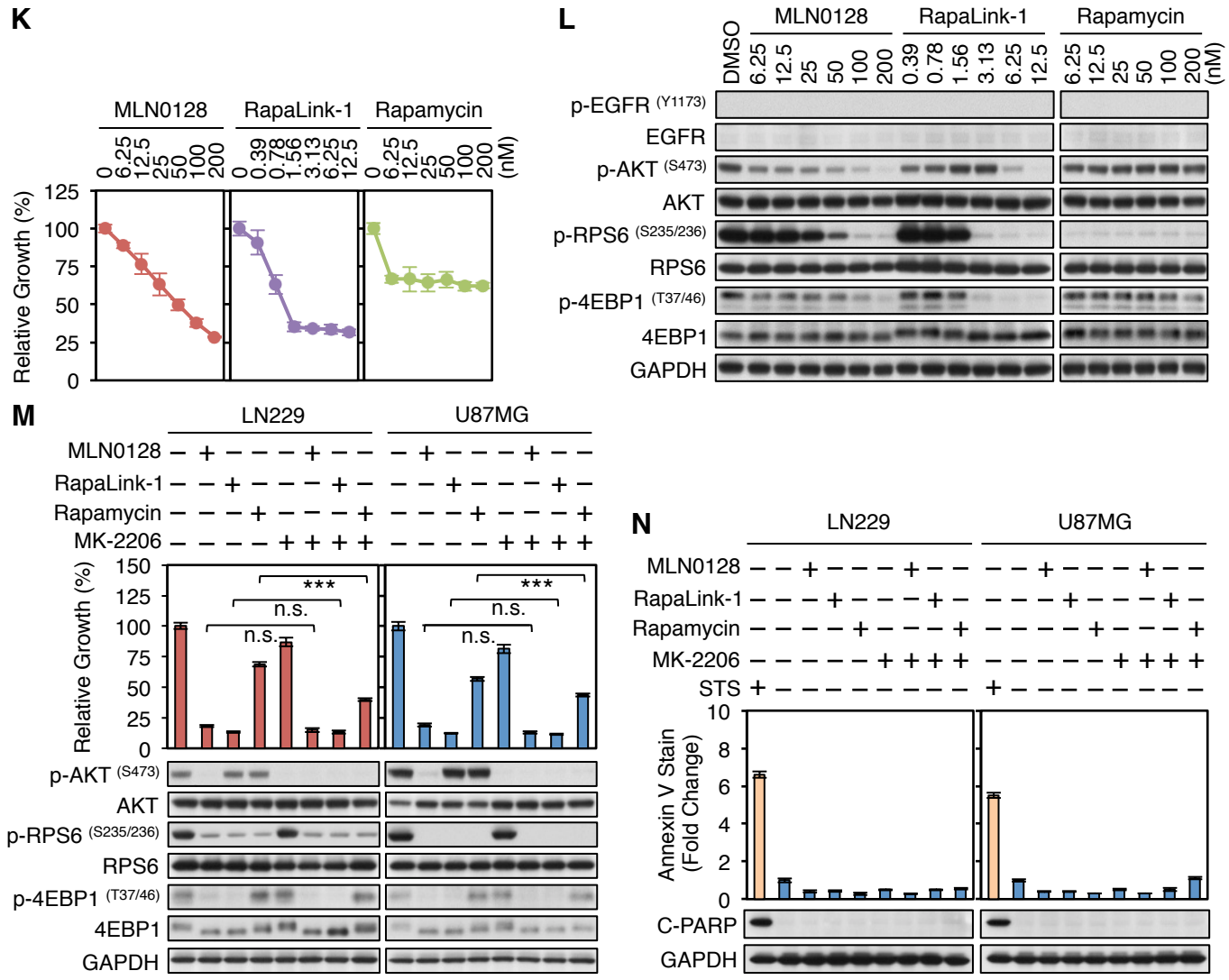

K
MLN0128
RapaLink-1
Rapamycin
0 6.25 12.5 25 50 100 200
0 0.39 0.78 1.56 3.13 6.25 12.5
0 6.25 12.5 25 50 100 200 (nM)
Relative Growth (%)
125
100
75
50
25
0
L
MLN0128
RapaLink-1
Rapamycin
DMSO 6.25 12.5 25 50 100 200 0.39 0.78 1.56 3.13 6.25 12.5 6.25 12.5 25 50 100 200 (nM)
p-EGFR (Y1173)
EGFR
p-AKT (S473)
AKT
p-RPS6 (S235/236)
RPS6
p-4EBP1 (T37/46)
4EBP1
GAPDH
M
LN229
U87MG
MLN0128
RapaLink-1
Rapamycin
MK-2206
Relative Growth (%)
150
125
100
75
50
25
0
***
n.s.
n.s.
p-AKT (S473)
AKT
p-RPS6 (S235/236)
RPS6
p-4EBP1 (T37/46)
4EBP1
GAPDH
N
LN229
U87MG
MLN0128
RapaLink-1
Rapamycin
MK-2206
STS
Annexin V Stain (Fold Change)
10
8
6
4
2
0
C-PARP
GAPDH

Figure S2 (related to Figure 2). RapaLink-1 inhibits the proliferation of multiple human GBM cell lines.
(A) Chemical structure of RapaLink-1 and -2.
(B) LN229 (top panels) and U87MG (bottom panels) cells were treated with doses of RapaLink-2 indicated for 3 days. Cell proliferation was measured by WST-1 assay. Data shown are means ± SD (Percentage growth relative to DMSO-treated control) of triplicate measurements.
(C) LN229 and U87MG cells were treated with doses of RapaLink-2 indicated for 3 hr, harvested, lysed, and analyzed by Western blotting as indicated. Representative blots from two independent experiments are shown.
(D) LN229 cells were treated with MLN0128, RapaLink-1, or rapamycin at doses indicated for 3 days. Proliferation was measured by WST-1 assay (top panels). Data shown are means ± SD (Percentage growth relative to DMSO-treated control) of triplicated measurements. Cells were treated as in top panels for 24 hr. Cell cycle was measured by flow cytometry. Percentage of cells in G0/G1, S, and G2/M phases is indicated in bottom panels. Data shown are means ± SD of triplicate measurements.
(E) Cells treated as in (D) for 3 hr were harvested, lysed, and analyzed by Western blotting as indicated. Cell lysates were from a single experiment. Gels were run for the same period of time, and blots were processed with equivalent exposure times, to assure reproducibility. Representative blots from three independent experiments are shown.
(F) LN229:EGFR, LN229:EGFRvIII, U87MG:EGFR, and U87MG:EGFRvIII cells were treated MLN0128, RapaLink-1, or rapamycin at indicated doses for 3 days. Proliferation was measured by WST-1 assay (top panels). Data shown are means ± SD (Percentage growth relative to DMSO-treated control) of triplicate measurements. Cells treated as in (top panels) for 3 hr were harvested, lysed, and analyzed by Western blotting (bottom panels) as indicated.
(G) Short term cultures from GBM43 (patient-derived glioma xenograft line) expressing firefly luciferase were treated with MLN0128, RapaLink-1, or rapamycin at indicated doses for 3 days. Luciferase activity was monitored by bioluminescence imaging (Luc-signal) after 3 days of treatment (top panels). Proliferation was measured by WST-1 assay (bottom panels). Data shown means ± SD (Percentage growth relative to DMSO-treated control) of triplicate measurements.
(H) Cells treated as in (G) for 3 hr were harvested, lysed, and analyzed by Western blotting as indicated. Cell lysates were from a single experiment. Gels were run for the same period of time, and blots were processed with equivalent exposure times, to assure reproducibility.
(I) Short term cultures from GBM5 (patient-derived glioma xenograft line) expressing firefly luciferase were treated with MLN0128, RapaLink-1, or rapamycin at indicated doses for 3 days. Luciferase activity was monitored by bioluminescence imaging (Luc-signal) after 3 days of treatment (top panels). Proliferation was measured by WST-1 assay (bottom panels). Data shown are means ± SD (Percentage growth relative to DMSO-treated control) of triplicate measurements.
(J) Cells treated for 3 hr as in (I) were harvested, lysed, and analyzed by Western blotting as indicated. Cell lysates were from a single experiment. Gels were run for the same period of time, and blots were processed with equivalent exposure times, to assure reproducibility.
(K) Short term cultures from GBM12 (patient-derived glioma xenograft line) were treated with MLN0128, RapaLink-1, or rapamycin at indicated doses for 3 days. Proliferation was measured by WST-1 assay. Data shown are means ± SD (Percentage growth relative to DMSO-treated control) of triplicate measurements.
(L) Cells treated for 3 hr as in (K) were harvested, lysed, and analyzed by Western blotting as indicated. Cell lysates were from a single experiment. Gels were run for the same period of time, and blots were processed with equivalent exposure times, to assure reproducibility.
(M) Parent LN229 and U87MG cells were treated with agents shown, singly or in combination for 3 days. Proliferation was measured by WST-1 assay. Data shown are means ± SD (Percentage growth relative to DMSO-treated control) of triplicate measurements (top panels). Cells treated as in (top panels) for 3 hr were harvested, lysed, and analyzed by Western blotting (bottom panels) as indicated.
(N) Parent LN229 and U87MG cells were treated as in (M) for 2 days or treated with 1 μM Staurosporine (STS) for 24 hr, apoptotic cells were analyzed by flow cytometry for Annexin V-FITC. Staurosporine (STS) served as a positive control for apoptosis. Data shown are means ± SD (fold change compared to DMSO-treated control) of triplicate measurements (top panel). An aliquot of cells was analyzed by Western blotting as indicated (bottom panel).

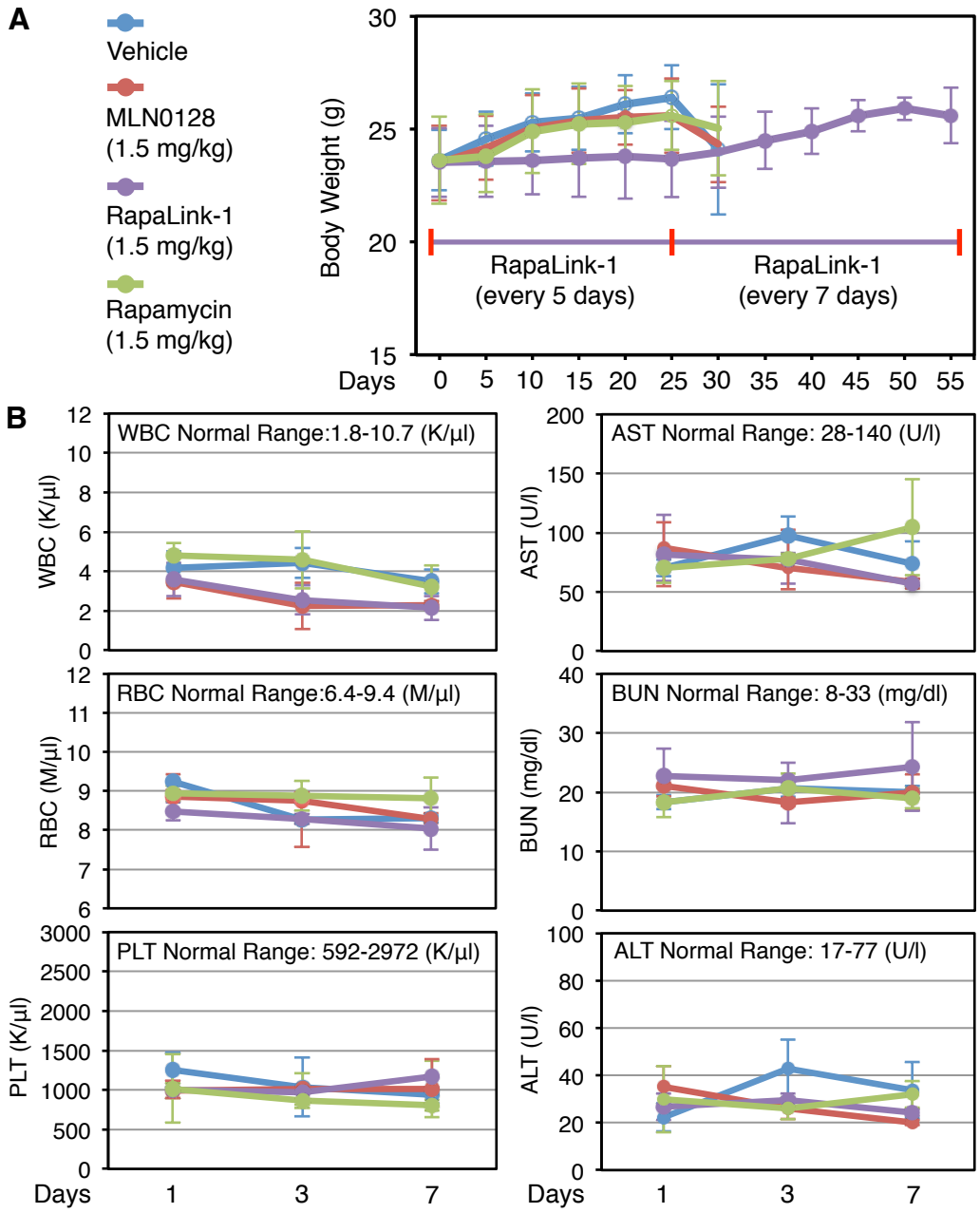


Figure S3 (related to Figure 3). Toxicity evaluation of mTOR inhibitors in vivo.

(A) BALB/C$^{nu/nu}$ mice bearing U87MG intracranial xenografts were treated on day zero with IP injections of vehicle (daily), MLN0128 (1.5 mg/kg, daily), rapamycin (1.5 mg/kg, daily) or RapaLink-1 (1.5 mg/kg, every five or seven days) at doses indicated. Body weights were measured every five days. Data shown are means ± SD of 9-12 mice in each group.

(B) Three BALB/C$^{nu/nu}$ mice from each group were treated as indicated doses in (A) on day zero with IP injections of vehicle (1.5 mg/kg, daily), MLN0128 (1.5 mg/kg, daily), rapamycin (1.5 mg/kg, daily), or RapaLink-1 (1.5 mg/kg, every five days), sacrificed on day one, three and seven. Complete Blood Count (CBC) and chemistries were analyzed. Data shown means ± SD of 3 mice in each group.

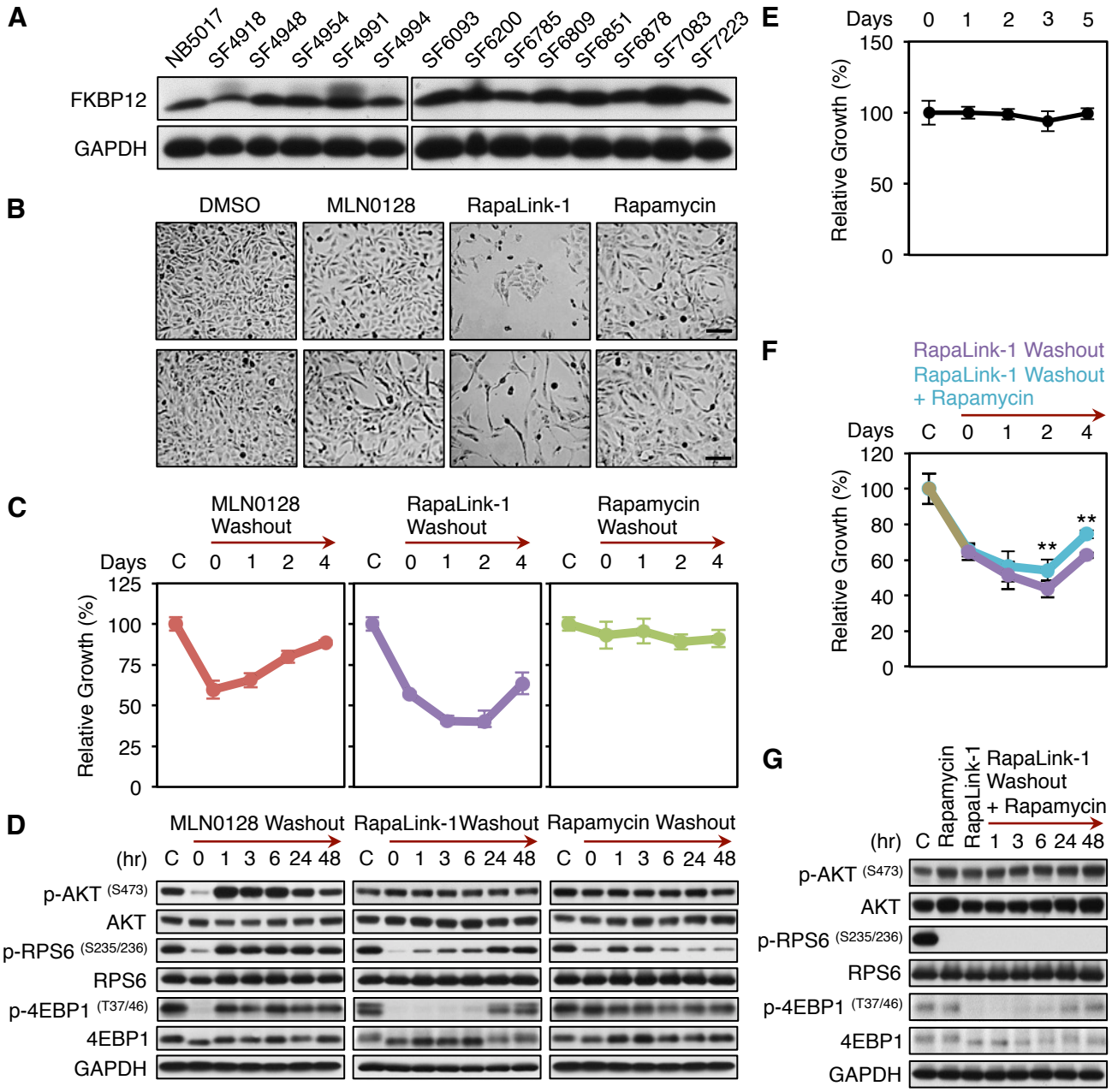

A
NB5017
SF4918
SF4948
SF4954
SF4991
SF4994
SF6093
SF6200
SF6785
SF6809
SF6851
SF6878
SF7083
SF7223
FKBP12
GAPDH
B
DMSO
MLN0128
RapaLink-1
Rapamycin
C
MLN0128 Washout
RapaLink-1 Washout
Rapamycin Washout
Days C 0 1 2 4
Relative Growth (%)
125
100
75
50
25
0
D
MLN0128 Washout
RapaLink-1Washout
Rapamycin Washout
(hr) C 0 1 3 6 24 48
p-AKT (S473)
AKT
p-RPS6 (S235/236)
RPS6
p-4EBP1 (T37/46)
4EBP1
GAPDH
E
Days 0 1 2 3 5
Relative Growth (%)
150
100
50
0
F
RapaLink-1 Washout
RapaLink-1 Washout
+ Rapamycin
Days C 0 1 2 4
Relative Growth (%)
120
100
80
60
40
20
0
**
**
G
Rapamycin
RapaLink-1
RapaLink-1
Washout
+ Rapamycin
(hr) C 1 3 6 24 48
p-AKT (S473)
AKT
p-RPS6 (S235/236)
RPS6
p-4EBP1 (T37/46)
4EBP1
GAPDH

Figure S4 (related to Figure 5). Effects of RapaLink-1 are durable.

(A) Normal brain (autopsy specimen, NB5017) or primary human glioblastoma tumors obtained from the Brain Tumor Research Center at UCSF were lysed and Western blotting as indicated. GAPDH is shown as loading control. Cell lysates were from a single experiment. Gels were run for the same period of time, and blots were processed with equivalent exposure times, to assure reproducibility.

(B) LN229 (top panels) and U87MG (bottom panels) cells were treated with 200 nM MLN0128, 1.56 nM RapaLink-1, or 10 nM rapamycin for 24 hr, followed by washout. Representative photographs were taken four days after inhibitors washout. Scale bar = 100 μm.

(C) LN229 cells were treated as in (B) for 24 hr, followed by washout for one to four days. Proliferation was measured by WST-1 assay. Data shown are means ± SD (Percentage growth relative to DMSO-treated control) of triplicated measurements.

(D) LN229 cells were treated as in (C) for 24 hr, followed by washout for 1-48 hr. Cells were harvested, lysed, and analyzed by Western blotting as indicated.

(E) U87MG cells were treated with 1 μM FK-506 for various times (1-5 days), proliferation was measured by WST-1 assay. Data shown are means ± SD (Percentage growth relative to DMSO-treated control) of triplicated measurements.

(F) U87MG cells were treated with DMSO, 1.56 nM RapaLink-1 for 24 hr (left two lanes). Cells treated with RapaLink-1 for 24 hr were resuspended in media with or without 10 nM rapamycin in the absence of RapaLink-1 for 1-4 days (right three lanes). Proliferation was measured by WST-1 assay. (On day 2, RapaLink-1 washout vs RapaLink-1 washout + rapamycin, **p = 0.0086; Day 4, RapaLink-1 washout vs RapaLink-1 washout + rapamycin, **p = 0.0021). Data shown means are ± SD (Percentage growth relative to DMSO-treated control) of triplicated measurements. C group represents DMSO treatment alone.

(G) U87MG cells were treated with DMSO, 10 nM rapamycin, or 1.56 nM RapaLink-1 for 24 hr (left three lanes). Cells treated with RapaLink-1 for 24 hr were resuspended in media with 10 nM rapamycin in the absence of RapaLink-1, and grown for 1-48 hr (right 5 lanes). Cells, were harvested, lysed, and analyzed by Western blotting as indicated. Representative blots from two independent experiments are shown. C group represents DMSO treatment alone.